Peer-Review

Korkmaz, Ela D, and Benan Temizci. 2025. “Coffee Bean-Derived Extracellular Vesicles Exhibit Anti-Cancer Effects on Neuroblastoma Cells.” *Journal of High School Science* 9 (3): 306–22. https://doi.org/10.64336/001c.143548.

This is a very well designed and reported study. Congratulations. I do however have some comments and concerns that need to be addressed.

1.Please report all centrifugation parameters in terms of RCF, For example you have reported this as RPM in section 2.5. In that section, you have stated that you centrifuged the cells twice more without reporting the centrifugation parameters. If they were the same, please explicitly state so.

2.I have a question re. tangential flow filtration. Did you use the retentate or filtrate as your primary source of exosomes? Should the retentate not have been used? Why then did you collect and concentrate the filtrate ? You do not report how much suspension was used and for what length of time through the tangential flow system. Please also report flow rate and the initial volume of suspension and final volumes of retentate and filtrate.

3.For the ELISA experiment, Similarly, you don’t report how non-specific binding was prevented in the ELISA, how unbound substrates were washed, how the unbound detection antibody was washed, how the signal was measured (fluorescence or absorbance? Instrument? Manufacturer?) How the standard curves were generated, what were the positive and negative controls? how many replicates? statistical analysis? I realize that you are only testing for the presence of TET8, however you have compared this protein’s expression in figure 2C for unroasted and roasted beans; hence you will need to present this data.

4.Was confluence determined visually?

5.You state “……In addition, the cells were treated with EV-depleted fractions of which volumes were determined by the highest concentration of coffee……..” I did not understand this statement. Please explicitly explain how much EV was in these negative controls. What was the objective of having EV being present in the negative controls?

6.Please report the source of the neuroblastoma cells, the unroasted and roasted coffee beans (vendor, expiration date, lot… as much information as you can possibly provide), Did you use an Annexin kit to detect apoptosis? If not, please provide sourcing of all materials (7AAD, Annexin V….).

7.How did you calculate EV particles per mL or microL ? from the particle size data in Figure 2 ? If yes, did you take the entire distribution into account or just the fraction with the greatest # of particles? Please describe in the manuscript.

8.It appears that both Alexa Fluor 647-conjugated Annexin-V as well as 7AAD emission lines are in the red Vis region. Why then do your cells appear blue and green in the flow cytometry analysis? Also, in the middle panel, I do not see any green stained cells. Can you please explain in the manuscript?

9.As you mention, coffee has a large content of secondary metabolites (not vesicle encapsulated) some of which are tumoricidal. What is the proportion of the tumoricidal effect generated by coffee EVs compared with the secondary metabolites, chlorogenic acid and caffeine ? Unless the magnitude of this

effect (EV/secondary metabolites) is large enough, the cost-benefit ratio of using EVs as tumoricidal agents may not materialize. Please discuss in the manuscript. It would have been interesting if you had compared the effect of coffee extract with and without EV content on neuroblastoma cells.

This is a very well designed and reported study. Congratulations. I do however have some comments and concerns that need to be addressed.

Thank you for your encouraging and constructive comments on my research study. I have addressed all the reviewers' comments and revised the manuscript accordingly. I sincerely appreciate the valuable feedback, which has given me the opportunity to improve the clarity and presentation of my work. All revisions are highlighted in blue. As new references have been added, the numbering of the reference list has also been updated, and these changes are likewise indicated in blue.

1. Please report all centrifugation parameters in terms of RCF, For example you have reported this as RPM in section 2.5. In that section, you have stated that you centrifuged the cells twice more without reporting the centrifugation parameters. If they were the same, please explicitly state so.
   Answer: All centrifugation parameters are revised to RCF. In section 2.5., additional centrifugation step parameters are also explicitly stated.

2. I have a question re. tangential flow filtration. Did you use the retentate or filtrate as your primary source of exosomes? Should the retentate not have been used? Why then did you collect and concentrate the filtrate ? You do not report how much suspension was used and for what length of time through the tangential flow system. Please also report flow rate and the initial volume of suspension and final volumes of retentate and filtrate.
   Answer: I used the retentate for extracellular vesicles (EVs) as they remained in the TFF device. Filtrate served as control since it was the EV-depleted fraction. More details including flow rate and the initial volume of suspension and final volumes of retentate and filtrate etc. have now been added to the Section 2.1 for isolation of EVs.

3. For the ELISA experiment, Similarly, you don't report how non-specific binding was prevented in the ELISA, how unbound substrates were washed, how the unbound detection antibody was washed, how the signal was measured (fluorescence or absorbance? Instrument? Manufacturer?) How the standard curves were generated, what were the positive and negative controls? how many replicates? statistical analysis? I realize that you are only testing for the presence of TET8, however you have compared this protein's expression in figure 2C for unroasted and roasted beans; hence you will need to present this data.
   Answer: Details related to the points raised regarding the ELISA experiment have now been added to Section 2.1.

4. Was confluence determined visually?
   Answer: As is standard in adherent cell culture, cell confluency was assessed visually using an inverted phase-contrast microscope. Detailed clarifications regarding this procedure have now been added to Section 2.2.

5. You state "……In addition, the cells were treated with EV-depleted fractions of which volumes were determined by the highest concentration of coffee……." I did not understand this statement. Please explicitly explain how much EV was in these negative controls. What was the objective of having EV being present in the negative controls?
   Answer: Thank you for pointing out the need for clarification. There were no EVs being present in the negative controls. The filtrate, EV-depleted fraction, was used as control to distinguish the effects mediated specifically by coffee-derived EVs from those potentially caused by soluble

components naturally present in the coffee extract. The EV-depleted fraction was obtained from the same coffee extract after ultracentrifugation, which effectively removed almost all EVs, leaving a negligible number of particles which might be coming from the solutes. Thus, the EV-depleted fraction might have contained the soluble secondary metabolites of coffee, with particle content reduced to an insignificant level.

For consistency, the volume of EV-depleted fraction applied to cells was matched to the volume of EV preparation used in the corresponding experimental group. For example, in the MTT assays, SH-SY5Y cells were treated with coffee EVs at final concentrations of 4×10^8, 6×10^8, and 8×10^8 particles/μL, which were prepared from the EV stock solution by adding 6.65, 9.97, and 13.3 μL per well, respectively. As a control, cells were treated with EV-depleted fraction at the same maximum volume used for EV treatment (13.3 μL). This ensured that the control group was exposed to the same amount of soluble coffee components, but without the functional contribution of EVs. Therefore, any observed differences between the EV-treated and EV-depleted control groups could be attributed primarily to the presence or absence of EVs, rather than other soluble constituents of coffee.

This possibly confusing sentence ("……In addition, the cells were treated with EV-depleted fractions of which volumes were determined by the highest concentration of coffee……..") and the control case are now revised to avoid confusion in the Section 2.3.

6. Please report the source of the neuroblastoma cells, the unroasted and roasted coffee beans (vendor, expiration date, lot… as much information as you can possibly provide), Did you use an Annexin kit to detect apoptosis? If not, please provide sourcing of all materials (7AAD, Annexin V….).

   Answer: The source, research resource identifier (RRID) number the neuroblastoma cells, and vendor of the coffee beans have now been added to the manuscript.

   The Annexin kit was not used to detect apoptosis. 7-AAD and Annexin-V were used separately, with their sources already listed as Beckman Coulter and BioLegend, respectively, in the manuscript. Their catalog numbers now have also been added to further clarify this point.
7. How did you calculate EV particles per mL or microL? from the particle size data in Figure 2 ? If yes, did you take the entire distribution into account or just the fraction with the greatest # of particles? Please describe in the manuscript.

   Answer: The number of EV particles/ml was calculated by the software of the NTA device. The ZetaView PMX-120 measures particle concentration and size by tracking the Brownian motion of individual particles in video frames, calculating their diffusion coefficients, and converting these into diameters using the Stokes–Einstein equation. Concentration is determined by counting detected particles within the instrument's calibrated viewing volume and adjusting for dilution, while size distribution comes from aggregating all calculated diameters into a histogram. The EV samples analyzed covered the entire distribution, approximately within the range of 50–200 nm.

   This information has now been added to the to the Section 2.1 in the manuscript.

8. It appears that both Alexa Fluor 647-conjugated Annexin-V as well as 7AAD emission lines are in the red Vis region. Why then do your cells appear blue and green in the flow cytometry analysis? Also, in the middle panel, I do not see any green stained cells. Can you please explain in the manuscript?

   Answer: The colored dots in flow cytometry pseudocolor graphs are generated by the FlowJo software and they illustrate the relative density of cells, not the spectrum they emit. Blue and green represent the areas of low cell density, red and orange represent the areas of high cell density, while yellow represents the areas of medium cell density.

This information has now been added in the manuscript to the Section 2.5.

9. As you mention, coffee has a large content of secondary metabolites (not vesicle encapsulated) some of which are tumoricidal. What is the proportion of the tumoricidal effect generated by coffee EVs compared with the secondary metabolites, chlorogenic acid and caffeine ? Unless the magnitude of this effect (EV/secondary metabolites) is large enough, the cost-benefit ratio of using EVs as tumoricidal agents may not materialize. Please discuss in the manuscript. It would have been interesting if you had compared the effect of coffee extract with and without EV content on neuroblastoma cells.
   Answer: We thank the reviewers for their comments, which highlighted that the manuscript did not clearly explain the rationale for focusing on coffee-derived extracellular vesicles (EVs) rather than coffee extracts or individual components, whose anticancer effects have already been well-documented in the literature.
   Previous studies have already reported the effects of coffee extracts (and other plant extracts prior to identification of extra-cellular vesicles) as well as purified coffee compounds. However, traditional plant extract-derived bioactive compounds, like those found in coffee, such as polyphenols, flavonoids, and alkaloids suffer from poor oral bioavailability, rapid degradation in the gastrointestinal tract, and low intestinal permeability, resulting in insufficient systemic exposure to exert therapeutic effects, whereas plant-derived extracellular vesicles offer an innovative solution by functioning as biocompatible, stable, and non-immunogenic delivery vehicles that encapsulate these bioactive molecules, protecting them from harsh digestive conditions and enzymatic breakdown while facilitating their efficient uptake by target cells (Sah et al, 2025). Hence, plant-derived extracellular vesicles offer a wide range of advantages compared to traditional plant extracts.
   In addition, recent evidence shows that secondary metabolites are also encapsulated in plant extracellular vesicles. There are currently reported metabolites in plant extracellular vesicles from important plant sources (Alfieri et al., 2021; Rizzo et al., 2021; Urzi et al., 2021). Moreover, plant extracellular vesicles contain higher concentrations of bioactive compounds compared to the extract forms, as stated in the manuscript) (Reference 20 in the manuscript: Langellotto et al., 2025).

   This additional information regarding the benefits of plant extracellular vesicles has now been added to the discussion section to provide a clearer context for the experimental approach.

   Since the anti-cancer effects of traditional coffee extracts have already been reported in the literature, and given the current evidence indicating the presence of all bioactive compounds including primary and secondary metabolites, and based on the knowledge that roasting changes the chemical and biochemical composition of coffee, in this study EVs isolated from unroasted and roasted Coffea arabica beans were chosen for investigation. Moreover, any available secondary metabolites that may not be encapsulated, would be present in EV-depleted fraction which was used as control condition to blank out from the effect of EV fractions. Furthermore, the results of the flow cytometry experiments conducted to analyze apoptotic cells indicate that the anticancer effects of coffee EVs (Unroasted and Roasted panels) are much greater than those of EV-depleted (Control panel) fraction, possibly containing free secondary metabolites, as there are more cells present in Q2 and Q3 quadrants representing late and early apoptosis, respectively in Unroasted and Roasted panels compared to Q2 and Q3 quadrants of the Control panel .

Additionally, while the free secondary metabolites, chlorogenic acid and caffeine, etc., require much higher doses to achieve significant effects in vitro, the concentrated and protected components within EVs may allow for more efficient intracellular delivery, and thereby translationally improving the cost-benefit ratio for potential therapeutic applications.
Furthermore, I am continuing the investigation of the effects of coffee EVs on other cancer cells and further molecular mechanisms of these effects, and developing results indicate that the anti-cancer effects of coffee EVs are not merely a result of their secondary metabolite content, but rather bioactive components like regulatory RNAs due to the stability advantages conferred by the vesicular structure.

Alfieri M, Leone A, Ambrosone A. Plant-derived nano and microvesicles for human health and therapeutic potential in nanomedicine. Pharmaceutics. 2021;13:498.

Rizzo J, Taheraly A, Janbon G. Structure, composition and biological properties of fungal extracellular vesicles. microLife. 2021.

Urzi O, Raimondo S, Alessandro R. Extracellular vesicles from plants: current knowledge and open questions. Int J Mol Sci. 2021;22:5366

Langellotto, M. D., Rassu, G., Serri, C., Demartis, S., Giunchedi, P., & Gavini, E. (2025). Plant-derived extracellular vesicles: a synergetic combination of a drug delivery system and a source of natural bioactive compounds. Drug delivery and translational research, 15(3), 831-845
Sah NK, Arora S, Sahu RC, Kumar D, Agrawal AK. Plant-based exosome-like extracellular vesicles as encapsulation vehicles for enhanced bioavailability and breast cancer therapy: recent advances and challenges. Medical Oncology. 2025 Jun;42(6):1-9.

Thank you for addressing my comments. Accepted. Please review the attached galley proof for errors and revert to the Journal in 48 hours to ensure a timely publication.